Journal of Chromatography B, 879 (2011) 1725–1731

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous determination of lipoic acid (LA) and dihydrolipoic acid (DHLA) in human plasma using high-performance liquid chromatography coupled with electrochemical detection

Abad Khan [a], Zafar Iqbal [a,*], David G. Watson [b], Amirzada Khan [a], Inamullah Khan [a], Naveed Muhammad [a], Salar Muhammad [a], Hashmat Ara Nasib [c], Naveed Iqbal [d], Faiz-ur-rehman [e], Muhammad Kashif [f]

[a] Department of Pharmacy, University of Peshawar, Peshawar -25120, Pakistan
[b] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow GI IXW. UK
[c] Khyber Teaching Hospital, Peshawar, Pakistan
[d] Hayatabad Medical Complex, Peshawar, Pakistan
[e] Kidney Centre, Peshawar, Pakistan
[f] Lady Reading Hospital, Peshawar, Pakistan

ARTICLE INFO

Article history:
Received 1 March 2011
Accepted 15 April 2011
Available online 22 April 2011

Keywords:
Lipoic acid
Dihydrolipoic acid
Liquid–liquid extraction
Plasma
Analysis
Oxidative stress

ABSTRACT

A fast, simple, and a reliable high-performance liquid chromatography linked with electrochemical detector (HPLC–ECD) method for the assessment of lipoic acid (LA) and dihydrolipoic acid (DHLA) in plasma was developed using naproxen sodium as an internal standard (IS) and validated according to standard guidelines. Extraction of both analytes and IS from plasma (250 μl) was carried out with a single step liquid–liquid extraction applying dichloromethane. The separated organic layer was dried under stream of nitrogen at 40 °C and the residue was reconstituted with the mobile phase. Complete separation of both compounds and IS at 30 °C on Discovery HS C18 RP column (250 mm × 4.6 mm, 5 μm) was achieved in 9 min using acetonitrile: 0.05 M phosphate buffer (pH 2.4 adjusted with phosphoric acid) (52:48, v/v) as a mobile phase pumped at flow rate of 1.5 ml min^{-1} using electrochemical detector in DC mode at the detector potential of 1.0 V. The limit of detection and limit of quantification for lipoic acid were 500 pg/ml and 3 ng/ml, and for dihydrolipoic acid were 3 ng/ml and 10 ng/ml, respectively. The absolute recoveries of lipoic acid and dihydrolipoic acid determined on three nominal concentrations were in the range of 93.40–97.06, and 93.00–97.10, respectively. Similarly coefficient of variations (% CV) for both intra-day and inter-day were between 0.829 and 3.097% for lipoic acid and between 1.620 and 5.681% for dihydrolipoic acid, respectively. This validated method was applied for the analysis of lipoic acid/dihydrolipoic acid in the plasma of human volunteers and will be used for the quantification of these compounds in patients with oxidative stress induced pathologies.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Alpha lipoic acid (ALA), is a derivative of octanoic acid and is also known as thioctic acid. Chemically it is, 1,2-dithiolane-3-valeric acid or 6,8-dithiolane octanoic acid, or 6,8-thioctic acid. It is found naturally in both plants and animals [1–4]. Alpha-lipoic acid (LA) and its reduced form dihydrolipoic acid (DHLA) is the crucial universal antioxidant redox couple of human body [4,5]. The chemical structures of LA and DHLA are presented in Fig. 1a and b, respectively.

The universal antioxidant redox couple of α-lipoic acid/dihydrolipoic acid plays the key role in human health by scavenging free radicals, chelating having metals, restoration of other antioxidants, and controlling regulatory proteins and genes essential for normal healthy life [6]. It is a cofactor for mitochondrial alphaketo-acid dehydrogenase, crucial prosthetic group of various cellular enzymes, chelating agent for heavy metals poisoning, and a scavenger of various free radicals such as hydroxyl radicals, hypochlorous acid, peroxyradicals, superoxide radicals, and singlet oxygen [1,2,5,7–11]. Lipoic acid restores others antioxidants of the body antioxidant network including glutathione, coenzyme Q10 and vitamins C and E to their reduced state and maintains body antioxidant capacity [4,12,13]. It is an effective therapeutic agent in so many diseases including diabetes, mitochondrial cytopathies, cardiovascular diseases, hepatitis, cataract, radiation damage, HIV infections, heavy metal poisoning, neurodegenerative disorders, and neurovascular abnormalities associated with diabetic neuropathy [3,14]. Due to its extensive

* Corresponding author. Tel.: +92 91 9239619; fax: +92 91 9218131.
E-mail address: zafar_iqbal@upesh.edu.pk (Z. Iqbal).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.04.017

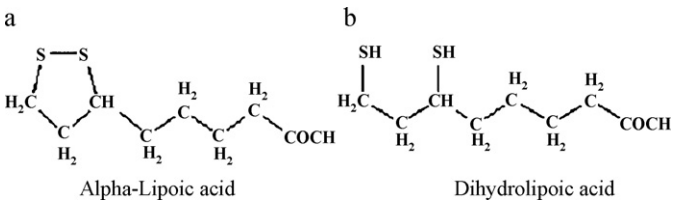

Fig. 1. (a) and (b) Chemical structures of LA & DHLA.

and multidisciplinary role in human health, a specific, quick and robust method of analysis is required that can be easily applied by standard research and clinical laboratories for simultaneous determination of its oxidized and reduced forms. The quantification of lipoic acid (both oxidized and reduced forms) is helpful in biochemical, nutritional and pharmacokinetic studies especially related to its homeostatic, and antioxidant role in human health [5].

Several chromatographic methods have been reported for the quantification of lipoic acid and its metabolites in pharmaceutical dosage forms [10,15], body fluids [1,5,16], and food samples [17]. Among the reported analytical methods for the determination of lipoic acid in the body fluids include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), polarography, colorimetric and microbiological assay, liquid chromatography mass spectrometry (LC-MS) [1,3,18,19], gas chromatography mass spectrometry (GC-MS) and capillary electrophoresis [20,21]. Though GC-MS has been reported to be a suitable and powerful tool for the quantification of LA in body fluids but it is expensive and its use is not common in most of the laboratories [5,15]. HPLC is one of the most suitable and proper method for quantification of lipoic acid in biological fluids owing to its high accuracy, sensitivity, and simplicity regarding sample preparation and treatment procedures in routine laboratory practice [22]. HPLC linked with ultraviolet [23], fluorescence [5,24,25] and electrochemical detectors [16,22,26,27] has been used for the evaluation of lipoic acid in food supplements and biological samples. The major demerits associated with HPLC-UV method are the absence of strong chromophore in lipoic acid and its comparatively lower sensitivity [15]. HPLC with fluorescence detection, offers a good method for the determination of α-lipoic acid in biological fluids but its lengthy and time consuming derivitization and sample preparation steps are laborious [5,24]. HPLC with electrochemical detection method is therefore seems to be an ultimate choice in terms of sensitivity, shorter analysis time and lower cost for most of the standard research laboratories to quantify α-lipoic acid in biological fluids in comparison with other literature reported methods [3,5,15,18–21,23,24]. To our knowledge only a few HPLC methods with fluorescence detection have been reported for the simultaneous determination of LA and DHLA in biological fluids and pharmaceuticals samples [5,13], and no HPLC–ECD method for the simultaneous determination of LA and DHLA in biological fluids has been reported so for.

The aim of our presented work was to develop a fast, sensitive and robust HPLC–ECD method for the simultaneous determination of LA and DHLA in human plasma with internal standard calibrated (IS) method. This suggested method is fast and inexpensive in comparison with other literature reported HPLC–ECD methods for the determination of lipoic acid in human body fluids [16,22]. The method was found suitable for the quantification of LA and DHLA in human plasma using a simple single step liquid–liquid extraction. The method can also be applied for routine analysis of LA and DHLA in pharmaceutical dosage forms and dietary supplements with suitable adjustment in the extraction technique.

2. Experimental

2.1. Chemicals and reagents

Alpha Lipoic acid (ALA), dihydrolipoic acid (DHLA) and retinyl palmitate were purchased from Sigma–Aldrich (Oslo, Norway). Naproxen sodium was a kind gift of Saydon Pharma Pvt. Ltd. (Peshawar, Pakistan). HPLC grade acetonitrile, methanol, and analytical grade potassium dihydrogen phosphate (KH_2PO_4), phosphoric acid (H_3PO_4), dichloromethane, ethyl acetate, diethylether and hydrochloric acid were also purchased from Sigma–Aldrich (Oslo, Norway). Distilled water prepared with Millipore (Milford, USA) distillation apparatus was used throughout the studies for preparation of solutions and buffer.

2.2. Equipments

The chromatographic analysis was performed using a Perkin Elmer HPLC system (Norwalk, USA) consists of a pump (series 200), on-line vacuum degasser (series 200), autosampler (series 200), column oven (series 200), linked by a PE Nelson network chromatography interface (NCI) 900 with a DECADE II Electrochemical Detector (Antec Leyden, Netherlands), that contains Flexcell flow cell of an effective volume of 0.5 μl and comprises of three electrode configuration with a working glassy carbon electrode, a HyREF reference electrode (REF) and the auxiliary electrode (AUX), KCl Ag/AgCl. The whole HPLC system was controlled by Perkin Elmer Total Chrom Workstation Software (version 6.3.1). The data was obtained and evaluated using this software. The separation was achieved through Discovery HS C18 RP-HPLC column (250 mm × 4.6 mm, 5 μm; Bellefonte, USA), guarded by a Perkin Elmer C_{18} (30 mm × 4.6 mm, 10 μm; Norwalk, USA) pre-column guard cartridge.

2.3. Chromatographic conditions

HPLC separation of LA, DHLA and IS was achieved using electrochemical detector in DC mode at detector applied potential of 1000 mV. The mobile phase comprised of acetonitrile and 0.05 M potassium dihydrogenphosphate (pH 2.4, adjusted with 85% phosphoric acid) in the ratio of (52:48, v/v), was applied at the flow rate of 1.5 ml/min maintaining the column oven and detector temperature at 30 °C. The sample (20 μl) was injected into HPLC system through autosampler.

2.4. Preparation of standard solutions

Stock solutions of LA, DHLA and naproxen sodium (IS) quality control standards each 1 mg/ml were prepared in the mobile phase. The stock solutions were further diluted with the mobile phase and dilutions in the range of 0.050–1 μg/ml and 0.500–10 μg/ml for LA and DHLA, respectively, were obtained keeping the concentration of the IS constant (1.0 μg/ml). These stock solutions were then stored at −20 °C until analysis.

2.5. Sample preparation

Blood samples were collected in the ethylene diamintetraacetic acid (EDTA) tubes from the healthy volunteers (Pharmacy Graduates in Department of Pharmacy, University of Peshawar, aged 20–25 years) who had given their informed consent. The whole study protocol has been approved by the ethical committee of Department of Pharmacy, University of Peshawar. The collected blood was centrifuged at 14,500 rpm (2000 × *g*) for 15 min at 4 °C to separate the plasma. The plasma was stored at −20 °C till

further analysis. Plasma samples were thawed at room temperature and volume (250 μl) was transferred to plastic eppendorf tube (≈2 ml), spiked with suitable concentrations of LA and DHLA to prepare dilutions in their respective concentration range of 0.05–1 μg/ml and 0.500–10 μg/ml. The volume (25 μl) of internal standard (10 μg/ml) was added to each eppendorf tube (≈2 ml) containing LA and DHLA spiked samples to make IS concentration 1 μg/ml in each sample. Protein precipitation was achieved with acetonitrile (500 μl), and after vortexing for 1 min the targets compounds were extracted with 1 ml dichloromethane through simple liquid–liquid extraction procedure. The resulting mixture was then vortexed and centrifuged at 1860 × *g* at −10 °C for 10 min, the organic layer was separated and dried under nitrogen at 40 °C. The dried residue was reconstituted with 250 μl mobile phase, vortexed and centrifuged again. The transparent supernatant was collected and a volume (20 μl) was injected into HPLC system through autosampler. The prepared samples were analysed and calibration curves were constructed in their respective calibration ranges.

2.6. Chromatographic conditions optimizations

The following chromatographic conditions were optimized for the quantification of oxidized and reduced lipoic acid using RP-HPLC–ECD system in isocratic mode.

2.6.1. Detector potential optimization

The detector potential was applied in the range of 600–1100 mV. The voltammogram was constructed between applied potential and detector response. The detector potential at which all the analytes showed optimal response was selected as the optimized potential for the simultaneous determination of both LA and DHLA.

2.6.2. Mobile phase optimization

Organic solvents including methanol, acetonitrile, tetrahydofurane (0.05%) and phosphate buffer (0.025–0.05 M) in different composition were applied for the separation of LA and DHLA and finally acetonitrile: 0.05 M phosphate buffer pH 2.4, adjusted with phosphoric acid, was selected for the analysis of both analytes. The peak responses of LA and DHLA were recorded against all combinations of acetonitrile: 0.05 M phosphate buffer (pH 2.4, adjusted with phosphoric acid) (50:50; 52:48; 55:45; 60:40). The mobile phase composition that eluted the target peaks in a shorter time with greater sensitivity and peak resolution was selected.

2.6.3. Flow rate optimization

For the selection of optimal flow rate, mobile phase was pumped at different flow rates in the range of 1–2 ml/min. The flow rate of the mobile phase that resulted better separation of targets compounds was selected for the simultaneous determination of LA and DHLA.

2.6.4. Temperature optimization

Temperature is an important parameter that greatly affects the chromatographic analysis. To study its effect on the analysis of studied compounds column oven temperature variations were evaluated in the range of 25–45 °C. The observed changes in the sensitivity, retention time, and peak resolution of targets compounds with respect to temperature were recorded from the resulted chromatograms of studied compounds.

2.6.5. Extraction solvent optimization

Extraction of lipoic acid (both oxidized and reduced) and IS from plasma samples was carried out through applying a simple single-step liquid–liquid extraction procedure. Liquid–liquid extraction was carried out using different organic solvents including ethyl acetate (EA), dichloromethane (DCM), diethyl ether (DEE), acetonitrile, and a 50:50 mixture of DCM:DEE to obtain the maximum recovery of both analytes and IS. Plasma samples 250 μl each were spiked with 0.5 and 1.0 μg/ml of LA and DHLA each and 1 μg/ml IS. These samples were then vortex-mixed for 10 min, and after extraction were analysed through HPLC. The recoveries were determined at two concentration levels of LA and DHLA each. The solvent that resulted better recoveries for all the analytes was chosen as extraction solvent.

2.6.6. Selection of internal standard

Various compounds including vitamin A palmitate, retinyl acetate, tocopherol acetate and naproxen sodium were assessed to be used as an internal standard. Among all these compounds the one that showed better resolution and instrumental response along with compatability to the studied analytes was chosen as internal standard. In the present method naproxen sodium has been used as an internal standard.

2.7. Method validation

In order to validate the proposed method various parameters such as specificity, accuracy, precision, sensitivity, linearity, recovery, limits of detection (LOD), limit of quantitation (LOQ), robustness, and stability of samples were evaluated.

The specificity of the proposed chromatographic method was evaluated through analysis of both the analytes in mobile phase, blank plasma, and 1:1 mixture containing 1 μg/ml each of LA, DHLA and naproxen sodium and plasma samples spiked with 1 μg/ml of each analyte and IS.

Percent recovery was used to determine the accuracy of the suggested method. The % recoveries at two concentration levels of all the analytes were determined through spiking the plasma (250 μl) with 0.5, and 1.0 μg/ml of each analyte and 1 μg/ml of IS in triplicate. Recoveries were determined according to the following equation:

$$\text{Recovery} = \frac{[C] \times 100}{[A] + [B]} \quad (1)$$

where A = response ratio of the analyte with respect to IS in the mobile phase; B = response ratio of analyte with respect to IS in the control plasma; C = response ratio of the analyte with respect to IS in spiked plasma.

To assess the linearity of the method calibration curves were constructed at six concentrations points of each analyte in the mobile phase and spiked plasma samples. Calibration curves were obtained by plotting the response ratios (ratios of peak areas of analyte to internal standard) of each analyte against its respective spiked concentrations using a linear least squares regression. The slope (m), intercept (b), and correlation coefficient (r) were calculated for each analyte from regression equation of its resulting plot.

Precision studies were carried out on the basis of injection repeatability and analysis repeatability of spiked plasma samples. Injection repeatability was evaluated through repeated injection (n = 10) of plasma sample spiked with 1 μg/ml of each analyte and internal standard into HPLC. The retention time and peak area repeatability data obtained as mean, standard deviation (±SD) and covariance (% RSD), was expressed as a measure of precision of the method. Analysis repeatability was evaluated through analysing plasma samples (n = 5) spiked with 1 μg/ml of each analyte and internal standard, prepared individually from same human plasma and the results obtained in terms of recovered amount were expressed by mean, standard deviation (±SD), and covariance (% RSD).

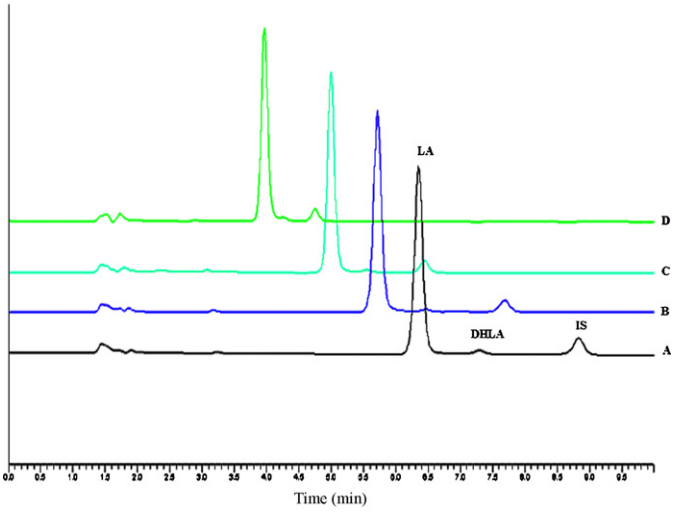

Fig. 2. Effect of mobile phase (ACN: KH_2PO_4) compositions on the elution of analytes.

The intra-day and inter-day studies were evaluated through analysing plasma samples spiked with appropriate amount of each analyte and IS (1 μg/ml), at 8:00, 16:00, and 24:00 h, for one week at three alternate days. The results were expressed as mean, standard deviation (±SD), and covariance (% RSD). The recovered amounts were calculated using the following equation:

$$C = \frac{X}{Y} \times \frac{A}{B} \times C_s \times F_D \quad (2)$$

where X and Y are peak areas of each analyte in plasma samples and 1:1 mixture, respectively; A and B are peak areas of the internal standard in 1:1 mixture and plasma samples, respectively; C_s is the concentration of analyte in the 1:1 mixture; and F_D is the dilution factor.

The sensitivity of the method was determined by quantifying the limit of detection (LOD) and limit of quantification (LOQ) for both LA and DHLA. The limit of detection (LOD) of the analyte is the concentration at which signal-to-noise ratio (S/N) is three and limit of quantification (LOQ) is the concentration of analyte that produced a response equal to 10 times the value of signal-to-noise ratio (S/N). For LOD and LOQ quantification dilutions of LA and DHLA were prepared in the range of 0.1–5 ng/ml and 1–20 ng/ml, respectively. The LOD and LOQ for both LA and DHLA were

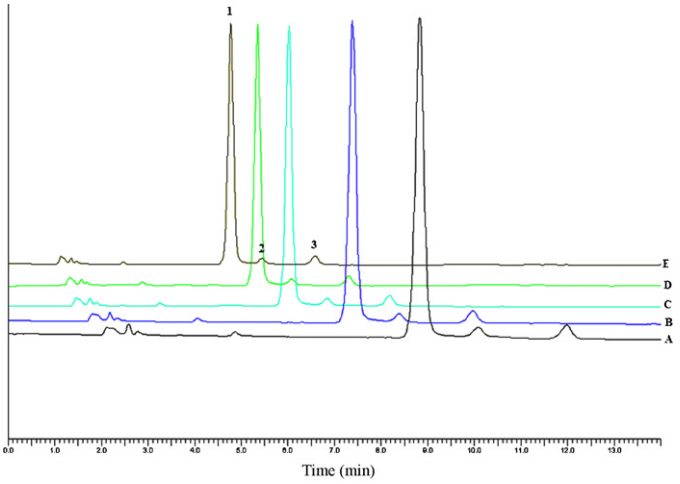

Fig. 3. Effect of mobile phase flow rates on the separation of analytes.

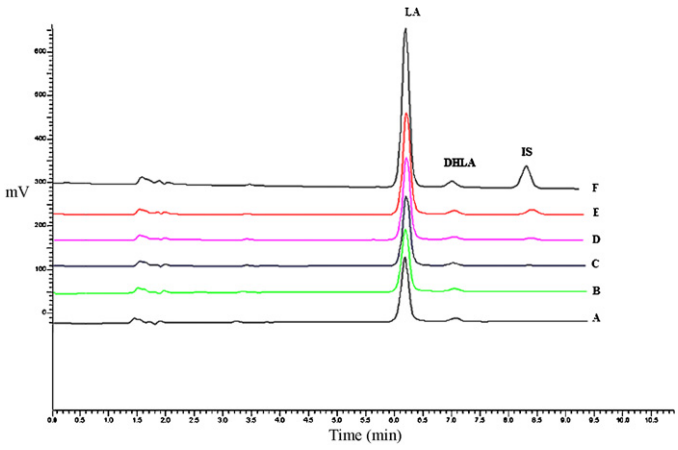

Fig. 4. Hydrodynamic voltammogram showing the effect of various applied detector electrode potentials on the peak response of LA, DHLA and IS.

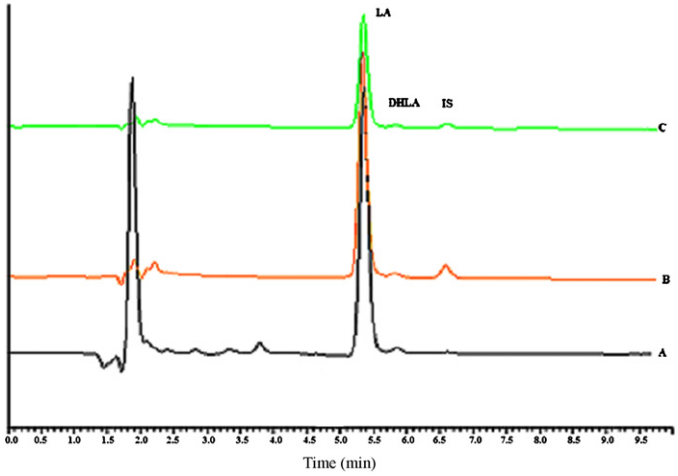

Fig. 5. Representative chromatograms showing LA, DHLA and IS in blank plasma (A), spiked plasma (B), and 1:1 mixture (C).

then determined from their respective chromatograms through software.

The robustness/ruggedness of the reported method was tested through small purposeful changes in the various chromatographic conditions, like mobile phase composition (±2%), column oven temperature (±5 °C), and flow rate of mobile phase (0.2 ml/min).

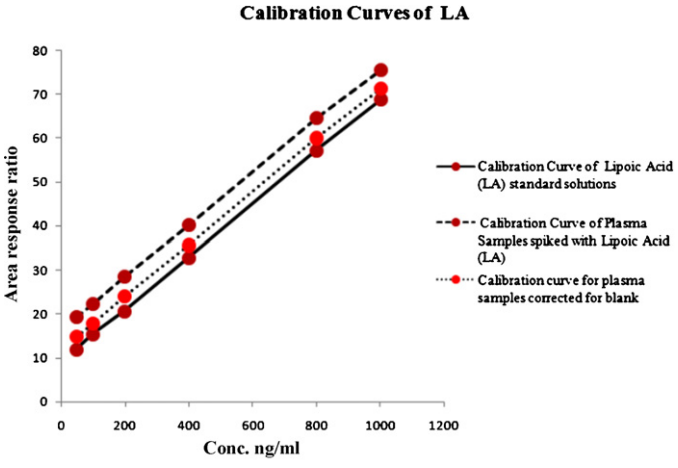

Fig. 6. Calibration curves of lipoic acid (LA).

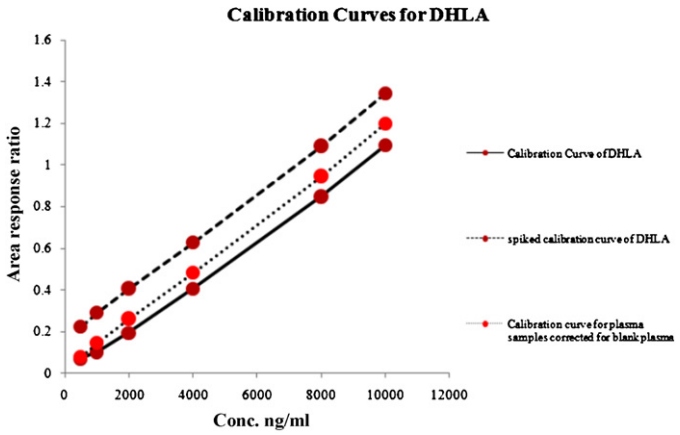

Fig. 7. Calibration curves of dihydrolipoic acid (DHLA).

Stability studies of LA and DHLA standard stock solutions and spiked plasma samples stored at 25 °C, 4 °C, and −20 °C were carried out for one month. The % stability was calculated by the following equation.

$$\%\ \text{Stability} = \frac{S_t}{S_0} \times 100 \quad (3)$$

where S_t is the stability of analyte at time t, and S_0 is the stability at initial time.

3. Results and discussion

The proposed method for the determination of LA and DHLA in human plasma using naproxen sodium as an internal standard is simple, fast, and reproducible. HPLC separation of LA, DHLA and naproxen sodium was obtained within 9 min with reasonable sensitivity. Some unknown compounds have been co-extracted with the applied extraction procedure without any interference with the target compounds. The proposed method was optimized regarding different chromatographic conditions and experimental parameters and validated in accordance with standard guidelines.

3.1. Optimization of chromatographic conditions and experimental parameters

The experimental parameters and chromatographic conditions were optimized for the proposed method and this method was found suitable for the simultaneous determination of LA and DHLA in human plasma. Mobile phase comprised of acetonitrile and 0.05 M phosphate buffer (pH 2.4 adjusted with phosphoric acid) in the ratio of (52:48, v/v), presented better resolution and sensitivity along with shorter retention times of analytes. The time of elution of all the analytes decreased by increasing the ratio of acetonitrile, however co-elution of LA and DHLA has been observed (Fig. 2).

Similarly, mobile phase flow rate was adjusted 1.5 ml/min as better peaks shape and separation of all the analytes and IS were observed at this flow rate. Retention times of both analytes decreased at higher flow rates however the peaks resolution decreased at higher flow rates as the peaks of LA and DHLA were co-eluted (Fig. 3).

The detector voltage of 1000 mV was chosen as the optimum potential for the evaluation of LA, DHLA, and IS as all these analytes resulted good instrumental response at this voltage. From the voltammogram it is apparent that highest instrumental responses for LA, DHLA and naproxen sodium were observed at 1000 mV. The responses of DHLA and naproxen sodium greatly decreased by lowering the detector voltage and below 700 mV these were not detectable as shown in Fig. 4. Optimization of column oven temperature was based on better sensitivity, good resolution, and shorter retention times of studied compounds (LA, DHLA and IS). The sensitivity and retention times of analytes and internal standard were greatly influenced by column oven temperature. Peak response increased and retention time decreased of both analytes and internal standard up to 30 °C, while above that temperature resolution of peaks decreased although there was a great reduction in the retention times of all the analytes. The peaks of both the com-

Table 1
Concentration range, linearity, accuracy, repeatability, recovery, and sensitivity of the developed method.

S/No.	Parameters	Lipoic acid (LA)	Dihydrolipoic acid (DHLA)
1	Concentration range (ng/ml)	0.050–1000	0.500–10,000
2	Linearity		
(a)	Standard solutions		
	Regression equation	$y = 0.059x + 16.42$	$y = 0.000x + 0.170$
	Correlation coefficient, r	0.999	0.999
(b)	Spiked plasma samples		
	Regression equation	$y = 0.059x + 11.85$	$y = 0.000x + 0.022$
	Correlation coefficient, r	0.999	0.999
(c)	Corrected plasma samples		
	Regression equation	$y = 0.059x + 9.07$	$y = 0.000x - 0.006$
	Correlation coefficient, r	0.999	0.998
3	Accuracy (% recovery)		
	0.5 μg/ml	96.50 ± 2.59; 2.68	97.41 ± 1.73; 1.78
	1.0 μg/ml	95.43 ± 1.56; 1.63	94.65 ± 2.03; 2.14
4	Precision		
	Repeatability		
(a)	Injection repeatability		
	Retention time (min)	5.80 ± 0.22; 3.79	6.5 ± 0.14; 2.15
	Peak area, 1 μg/ml	2,168,094 ± 25,664; 1.18	40,468 ± 1536; 3.79
(b)	Analysis repeatability		
	(amount recovered, 1 μg/ml)	0.9424 ± 0.0182; 1.93	0.8972 ± 0.0165; 1.84
5	Recovery (amount recovered, μg/ml)		
	0.5 μg/ml	0.476 ± 0.015; 3.15 (95.20%)	0.465 ± 0.007; 1.50 (93.00%)
	1.0 μg/ml	0.934 ± 0.014; 1.49 (93.40%)	0.917 ± 0.026; 2.84 (97.10%)
	5.0 μg/ml	4.853 ± 0.241; 4.96 (97.06%)	4.675 ± 0.218; 4.66 (93.5%)
6	Sensitivity		
(a)	Limit of detection (ng/ml)	0.500	3
(b)	Limit of quantification (ng/ml)	3.000	10

Table 2
Intra-day and inter-day studies performed for Lipoic acid and Dihydrolipoic acid.

S/No.	Parameters	Alpha-lipoic acid	Dihydrolipoic acid
		Mean ± SD; % CV	Mean ± SD; % CV
1	Intra-day repeatability		
	0.5 μg/ml (amount recovered, μg/ml)	0.473 ± 0.014; 2.959	0.453 ± 0.019; 4.194
	0.8 μg/ml (amount recovered, μg/ml)	0.765 ± 0.018; 2.352	0.778 ± 0.013; 1.670
	1 μg/ml (amount recovered, μg/ml)	0.965 ± 0.008; 0.829	0.864 ± 0.014; 1.620
2	Inter-day repeatability		
	0.5 μg/ml (amount recovered, μg/ml)	0.452 ± 0.014; 3.097	0.442 ± 0.012; 2.714
	0.8 μg/ml (amount recovered, μg/ml)	0.765 ± 0.019; 2.483	0.734 ± 0.016; 2.179
	1 μg/ml (amount recovered, μg/ml)	0.864 ± 0.023; 2.662	0.792 ± 0.045; 5.681

Table 3
Stability study of LA and DHLA at various temperatures showing percent loss.

Analytes	Stability studies showing percent loss (% loss)		
Standard solutions[a]	Room temperature (25 °C)	Refrigerator temperature (4 °C)	Freezer temperature (−20 °C)
Lipoic acid (LA)	4.79	2.48	1.16
Dihydrolipoic acid (DHLA)	12.74	9.79	5.53
Spiked plasma samples[a]			
Lipoic acid (LA)	6.52	2.94	2.24
Dihydrolipoic acid (DHLA)	16.86	11.87	7.65

[a] *Note*: The standard solutions and spiked plasma samples of LA and DHLA were stored for one month.

pounds were sharp at 30 °C and the resolution of peaks decreased above that temperature. The peaks broadening may be due weakening of bonds between analytes and stationary phase or some other configurational changes in the molecules that occurred at higher temperature.

Internal standard selection was based on its sensitivity, specificity, stability and compatibility with the studied compounds and extraction procedure. Among all tested compounds as internal standard naproxen sodium showed the better results in terms of sensitivity, good recovery and retention time in comparison with the other internal standards used. Liquid–liquid extraction with various organic solvents was applied for the extraction of the above studied compounds from plasma and a single-step extraction with dichloromethane was selected as better recoveries of all the analytes were obtained.

3.2. Method validation

The reported HPLC method was validated using standard guidelines. Our suggested method was accurate and fully validated for the determination of both (reduced and oxidized) forms of lipoic acid in plasma using electro chemical detector. The separation of the target analytes was obtained in 9 min, through analysis of standard solutions, blank plasma and spiked plasma samples. Representative chromatograms of blank plasma, spiked plasma and 1:1 mixture (1 μg/ml of LA, DHLA and naproxen sodium each) in mobile phase are shown in Fig. 5A–C, respectively.

The calibration curves of LA and DHLA standard solutions and spiked plasma samples showed good linearity in the range of 0.05–1 μg/ml and 0.50–10 μg/ml, respectively. The calibration curves of LA and DHLA standard solutions, spiked plasma samples, and corrected samples for blank plasma constructed at six concentration levels, are shown in Figs. 6 and 7, respectively. Regression equation and correlation co-efficient (r) acquired from the calibration curves of LA and DHLA standard solutions, spiked plasma samples, and spiked plasma samples corrected for blank plasma were; $y = 0.059x + 16.42$, $r = 0.999$, $y = 0.059x + 11.85$, $r = 0.999$, $y = 0.059x + 9.07$, $r = 0.999$, and $y = 0.000x + 0.170$, $r = 0.999$, $0.000x + 0.022$, $r = 0.999$, $0.000x - 0.006$, $r = 0.998$, respectively, as shown in Table 1.

Percent recovery at 0.5, and 1 μg/ml concentration levels was determined to assess the accuracy of the method and was found 96.50, and 95.43, for LA and 97.41, and 94.65, for DHLA, respectively, as shown in Table 1. The injection repeatability, analysis repeatability, and intra-day, inter-day studies for both the analytes are presented in Tables 1 and 2, respectively. The intra-day precision (% RSD) at 0.5 μg/ml, 0.8 μg/ml, and 1 μg/ml were 2.959, 2.352, and 0.829, for LA and 4.194, 1.670, and 1.620, for DHLA, respectively. The inter-day precisions (%RSD) were 3.097, 2.483, and 2.662 for LA and 2.714, 2.179, and 5.681, for DHLA at 0.5, 0.8 and 1 μg/ml, respectively (Table 2).

Recoveries of both analytes were measured at three concentrations using liquid–liquid extraction. The % recoveries for LA and DHLA using liquid–liquid extraction were in the range of 93.40–97.06% and 93.0–97.10%, respectively as shown in Table 1. The recovery of IS determined at a single concentration of 1 μg/ml was 94.70%.

LOD and LOQ values for both LA and DHLA were evaluated to assess the sensitivity. The LOD and LOQ values were 0.500 and

Table 4
Plasma Lipoic acid (LA) and dihydrolipoic acid (DHLA) profile of Healthy Volunteers ($n = 15$)[a].

Endogenous plasma LA and DHLA values obtained from healthy volunteers ($n = 15$)			
Analytes	Plasma concentration	Minimum value	Maximum value
Concentration (ng/ml)	Mean ± SD; %RSD		
Lipoic acid (LA)	35 ± 5.64; 16.11	29.47	40.66
Dihydrolipoic acid (DHLA)	173 ± 4.26; 2.46	168.75	177.47

[a] Age of volunteers was in the range of 22–25 years.

3.00 ng/ml, for LA and 3 and 10 ng/ml for DHLA, respectively as presented in Table 1.

The stability studies showed that the standard solutions and spiked plasma samples of both LA and DHLA were stable for at least one month when stored at −20 °C. Both standard solutions and spiked plasma samples of DHLA were degraded rapidly in comparison with LA standard and spiked samples as shown in Table 3.

The robustness/ruggedness of the reported method was tested through small purposeful changes in chromatographic conditions and the changes in the analysis of these analytes were found negligible.

4. Application of the method

Lipoic acid both in oxidized and reduced form is the crucial element of body extracellular antioxidant defense system. The accurate evaluation of plasma lipoic acid and the ratio of LA/DHLA will provide an indication that antioxidant intervention is necessary. Our suggested and validated HPLC–ECD method will be applied for the assessment of oxidative stress through measuring plasma concentration of lipoic acid (oxidized and reduced) in healthy volunteers and patients with diabetes and cardiovascular diseases. The suggested method can be easily applied for the determination of LA/DHLA in clinical set up and basic research studies to determine the etiologies of oxidative stress induced diseases. Similarly, the method can also be used as a tool to evaluate the mechanism and effectiveness of nutritional/dietary intervention strategies. The data acquired from healthy volunteers showed best peaks resolution, and the plasma values of lipoic acid were found in permissible range as shown in Table 4. This suggested HPLC method can also be applied for the quantification of lipoic acid in foods, food supplements, pharmaceutical dosage forms and others biological samples if adequate modifications are made in the extraction procedure used for these compounds.

5. Conclusion

The reported optimized HPLC–ECD method for the determination of oxidized and reduced forms of lipoic acid in human plasma was fast, simple, economical, accurate, sensitive, precise, selective and reproducible. The method was optimized regarding different chromatographic conditions and validated according to standard guidelines [28]. The reported method was validated on the basis of specificity, sensitivity, linearity, stability, precision, recovery, robustness and system suitability. The reported method is rapid (short analysis time), precise, accurate, economical and is based on single step liquid–liquid extraction procedure in comparison with other reported methods [16,22]. The reported method was successfully applied for the determination of plasma lipoic acid (oxidized and reduced) concentration in human volunteers as shown in Table 4. This method can also be found quite suitable for the investigation of lipoic acid in biological matrices as well as in pharmacokinetic studies.

Acknowledgement

We are grateful to Higher Education Commission of Pakistan (HEC) for financial support to carry out this project.

References

[1] A. Khan, M.I. Khan, Z. Iqbal, L. Ahmad, Y. Shah, D.G. Watson, J. Chromatogr. B 878 (2010) 2782.
[2] J. Bustamante, J.K. Lodge, L. Marcocci, H.J. Tritschler, L. Packer, B.H. Rihn, Free Radic. Biol. Med. 24 (1998), 1023. BB.
[3] R. Trivedi, R. Kallem, R. Mamidi, R. Mullangi, N. Srinivas, Biomed. Chromatogr. 18 (2004) 681.
[4] S.E.J. Seybolt, Med. Hypotheses 75 (2010) 572.
[5] A. Haj-Yehia, P. Assaf, T. Nassar, J. Katzhendler, J. Chromatogr. A 870 (2000) 381.
[6] H. Moini, L. Packer, N-E.L. Saris, Toxicol. Appl. Pharmcol. 182 (2002) 84.
[7] P. Arivazhagan, P. Juliet, C. Panneerselvam, Pharmacol. Res. 41 (2000) 299.
[8] L. Packer, E. Witt, H. Tritschler, Free Radic. Biol. Med. 19 (1995) 227.
[9] F. Navari-Izzo, M.F. Quartacci, C. Sgherri, Plant Physiol. Biochem. 40 (2002) 463.
[10] É. Kozlov, I. Solunina, M. Lyubareva, M. Nadtochii, Pharm. Chem. J. 38 (2004) 642.
[11] L. Packer, K. Kraemer, G. Rimbach, Nutrition 17 (2001) 888.
[12] C. DellaCroce, G. Bronzetti, M. Cini, L. Caltavuturo, G. Poi, Toxicol. In Vitro 17 (2003) 753.
[13] S. Satoh, T. Toyooka, T. Fukushima, S. Inagaki, J. Chromatogr. B 854 (2007) 109.
[14] S. Jain, G. Lim, Free Radic. Biol. Med. 29 (2000) 1122.
[15] A.I. Durrani, H. Schwartz, W. Schmid, G. Sontag, J. Pharm. Biomed. Anal. 45 (2007) 694.
[16] J. Teichert, R. Preiss, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 769 (2002) 269.
[17] W. Siangproha, P. Rattanaratb, O. Chailapakul, J. Chromatogr. A 1217 (2010) 7699.
[18] H.T. Chng, L.S. New, A.H. Neo, C.W. Goh, E.R. Browne, E.C.Y. Chan, J. Pharm. Biomed. Anal. 51 (2010) 754.
[19] J. Chen, W. Jiang, J. Cai, W. Tao, X. Gao, X. Jiang, J. Chromatogr. B 824 (2005) 249.
[20] H. Kataoka, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 717 (1998) 247.
[21] A. Sitton, M.G. Schmid, G. Gubitz, H.Y. Aboul-Enein, J. Biochem. Biophys. Methods 61 (2004) 119.
[22] J. Teichert, R. Prei, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 672 (1995) 277.
[23] H.Y. Aboul-Enein, H. Hoenen, J. Liq. Chromatogr. Related Technol. 27 (2004) 3029.
[24] W. Witt, B. Rüstow, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 705 (1998) 127.
[25] T. Inoue, M. Sudo, H. Yoshida, K. Todoroki, H. Nohta, M. Yamaguchi, J. Chromatogr. A 1216 (2009) 7564–7569.
[26] G.K. Ziyatdinova, G.K. Budnikov, V.I. Pogoreltsev, J. Anal. Chem. 59 (2004) 288.
[27] C. Sen, S. Roy, S. Khanna, L. Packer, Methods Enzymol. 299 (1999) 239.
[28] N.A. Epshtein, Pharma, Chem. J. 38 (2004) 212.